# MUTANT-TYPE BIOLUMINESCENT PROTEIN, AND PROCESS FOR PRODUCING THE MUTANT-TYPE BIOLUMINESCENT PROTEIN



What is claimed is:

1. A bioluminescent protein with improvements in catalytic efficiency or stability.

2. A bioluminescent protein according to claim 1, wherein the improvements includes at least one of 5 kinds of improvements in substrate specificity and maximum reaction rate in respect of catalytic efficiency, and thermal stability, pH stability and stability at low ion concentration in respect of stability.

3. A bioluminescent protein according to claim 1 or 2, which is a luciferase derived from beetles (Coleoptera).

4. A bioluminescent protein according to claim 1 or 2, which is a luciferase derived from fireflies.

5. A process for producing the mutant-type bioluminescent protein of claim 1 or 2, which comprises modifications to a bioluminescent protein precursor.

6. A process for producing the mutant-type bioluminescent protein of claim 1 or 2, wherein said modifications involve the replacement, alternation, removal and addition of at least one amino acid and the fusion of a plurality of proteins.

Example 1

10 $\mu$ g plasmid pT3/T7-LUC (obtained from Clontech) for expression of luciferase derived from an American firefly (Photinus pyralis) was added to 50 $\mu$ l restriction enzyme buffer K [20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol] and then cleaved with 20 U each of restriction

enzymes SphI and SmaI (Takara Shuzo Co., Ltd.) at 37 ℃ for 2 hours. This reaction solution was subjected to 0.8 % low-melting agarose gel electrophoresis, and a gel containing an about 1.1-kb DNA fragment containing a C-terminal portion of a luciferase gene derived from Photinus pyralis was cut off and then molten by heating at 65 ℃ for 5 minutes. To the molten gel was added a 2-fold volume of TE buffer [10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA], and after an equal volume of phenol saturated with TE buffer was added thereto, the mixture was stirred. After centrifugation at 12,000 r.p.m. for 15 minutes, the aqueous layer was recovered and then precipitated with a 2-fold volume of cold ethanol to recover the DNA fragment containing the C-terminal portion of the luciferase gene derived from Photinus pyralis.

Separately, synthetic DNAs (SEQ ID NO:1, CTC TAG CAT GCG AAA ATC TAG; SEQ ID NO:2, CTG CAG GCC TGC AAG CTT GG) [prepared by System 1 Plus DNA synthesizer, Beckman] was added to plasmid pGLf37 (described in Japanese Patent Laid-Open Publication No. 244,942/1993) for expression of luciferase derived from Genji firefly (Luciola cruciata), and polymerase chain reaction (PCR) was carried out as follows. 50 $\mu$ l PCR reaction solution contained 20 $\mu$ g plasmid pGLf37, 50 pmol each of the synthetic DNAs, 120 mM Tris-HCl (pH 8.0), 6 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.5 mM $MgSO_4$, 0.1 % Triton X-100, 0.001 % BSA, 0.2 mM each of dATP, dGTP, dCTP and dTTP, and 2.5 U of KOD DNA polymerase (Toyobo Co., Ltd.). This mixture was subjected to 25 cycles of PCR, each cycle consisting of incubation at 98 ℃ for 15 seconds, 65 ℃ for 2 seconds and 74 ℃ for 30 seconds in Perkin-Elmer Thermal Cycler PJ2000. To the reaction mixture was added an equal volume of phenol saturated with TE buffer, and the mixture was stirred. After centrifugation

at 12,000 r.p.m. for 15 minutes, the aqueous layer was recovered and then precipitated with a 2-fold volume of cold ethanol to recover the DNA fragment. It was dissolved again in TE buffer, then cleaved with SphI and subjected to low-melting agarose gel electrophoresis to recover an about 3.4-kbp DNA fragment containing an N-terminal portion of the luciferase gene derived from Luciola cruciata.

50 ng of the above SphI-SmaI fragment from pT3/T7-LUC and 50 ng of the above SphI-cleaved fragment from pGLf37 were incubated at 15 ℃ for 16 hours in 20 μl DNA ligase buffer in the presence of 300 U of T4 DNA ligase. The reaction mixture was used to transform E. coli JM109 (Toyobo Co., Ltd.) by the Hana-han method [DNA Cloning, 1, 109-135 (1985)], and ampicillin-resistant colonies were selected. A plasmid was removed from the formed colonies by the alkali-SDS method, and the structure of the plasmid was confirmed. This plasmid was subjected to reaction with a dye primer tuck sequencing kit (Applied Biosystems) and analyzed by electrophoresis with an ABI373 DNA sequencer (Applied Biosystems) to determine its nucleotide sequence. The determined nucleotide sequence is shown in SEQ ID NO:6, and the amino acid sequence of a polypeptide translated from said nucleotide sequence is shown in SEQ ID NO:5. The plasmid thus obtained was designated pGA1.

Plasmid pGA1 was used to transform E. coli JM109 in the manner described above to give E. coli JM109 (pGA1). E. coli JM109 (pGA1) was deposited as FERM BP-5990 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan. E. coli JM109 (pGA1) was inoculated on an LB-amp agar plate [1 % (W/V) Bacto-trypton, 0.5 % (W/V) yeast

extract, 0.5 % (W/V) NaCl, (50 $\mu$ g/ml) ampicillin and 1.4 % (W/V) agar] and cultured at 37 ℃. The colony microorganisms appearing 16 hours thereafter were inoculated into 10 ml of an LB-amp medium [1 % (W/V) Bacto-trypton, 0.5 % (W/V) yeast extract, 0.5 % (W/V) NaCl and (50 $\mu$ g/ml) ampicillin] and cultured at 37 ℃ for 18 hours under shaking. This culture, 10 ml, was inoculated into 2 L of the above LB-amp medium and cultured at 30 ℃ for 6 hours under shaking and then centrifuged at 8000 r.p.m. for 10 minutes to give 30 g wet microbial pellet. The recovered microorganism was suspended in 20 ml buffer consisting of 0.1 M $KH_2PO_4$ (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol and 0.2 mg/ml protamine sulfate, and 2 ml of 10 mg/ml lysozyme solution was further added thereto and the mixture was allowed to stand on ice for 15 minutes.

Then, this suspension was frozen in an ethanol/dry ice bath and then allowed to stand at a temperature of 25 ℃ until it was completely thawed. Further, it was centrifuged at 12,000 r.p.m. for 5 minutes whereby 20 ml crude enzyme was obtained as the supernatant. The crude enzyme solution thus obtained was purified according to the method described in Japanese Patent Laid-Open Publication 141592/1989, and the purified enzyme was designated GA1 luciferase. The Km value of this purified preparation for the substrate ATP was determined. The peak of the emission of light generated by use of the enzyme with the concentration of ATP varying from 0 to 2 mM in a solution containing 50 mM HEPES (pH 7.5), 0.2 mM luciferin and 10 mM $MgSO_4$ was measured in Luminometer ML3000 (Dynatech) and the result is shown in the table 1 below. The thus determined affinity of the GA1 luciferase for ATP was about 5.73-fold higher than the wild-type Photinus pyralis luciferase and about 11.4-fold higher than the wild-type Luciola

cruciata luciferase. This improvement in the affinity of the GA1 luciferase for ATP as compared with that of the wild-type luciferases revealed that the GA1 luciferase is a highly useful enzyme.

Table 1

| | Km (mM) |
|---|---|
| Photinus pyralis luciferase | 0.152 |
| Luciola cruciata luciferase | 0.301 |
| GA1 luciferase | 0.0265 |

Example 2

10 μg plasmid pT3/T7-LUC (obtained from Clontech) for expression of luciferase derived from an American firefly (Photinus pyralis) was added to 50 μl buffer H [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol] and then cleaved with 20 U each of restriction enzymes EcoRV and SalI (Takara Shuzo Co., Ltd.) at 37 ℃ for 2 hours. This reaction solution was subjected to 0.8 % low-melting agarose gel electrophoresis, and a gel containing an about 0.5-kb DNA fragment containing a C-terminal portion of a luciferase gene derived from Photinus pyralis was cut off and then molten by heating at 65 ℃ for 5 minutes. To the molten gel was added a 2-fold volume of TE buffer [10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA], and after an equal volume of phenol saturated with TE buffer was added thereto, the mixture was stirred. After centrifugation (12,000 r.p.m. for 15 minutes), the aqueous layer was recovered and then precipitated with a 2-fold volume of cold ethanol to recover a DNA fragment containing a region coding for the C-terminal of luciferase from Photinus pyralis.

Separately, synthetic DNAs (SEQ ID NO:3, ATC CTT TGT ATT TGA

TTA AAG; SEQ ID NO:4, TCT AGA GTC GAC CTG CAG GC) [prepared by System 1 Plus DNA synthesizer, Beckman] was added to plasmid pGLf37 T-M-2 (described in Japanese Patent Laid-Open Publication No. 244,942/1993) for expression of thermostable luciferase derived from Genji firefly (Luciola cruciata), and polymerase chain reaction (PCR) was carried out as follows. 50 μl PCR reaction solution contained 20 μg plasmid pGLf37 T-M-2, 50 pmol each of the synthetic DNAs, 120 mM Tris-HCl (pH 8.0), 6 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.5 mM $MgSO_4$, 0.1 % Triton X-100, 0.001 % BSA, 0.2 mM each of dATP, dGTP, dCTP and dTTP, and 2.5 U of KOD DNA polymerase (Toyobo Co., Ltd.). This mixture was subjected to 25 cycles of PCR, each cycle consisting of incubation at 98 ℃ for 15 seconds, 65 ℃ for 2 seconds and 74 ℃ for 30 seconds in Perkin-Elmer Thermal Cycler PJ2000. To the reaction mixture was added an equal volume of phenol saturated with TE buffer, and the mixture was stirred. After centrifugation (12,000 r.p.m. for 15 minutes), the aqueous layer was recovered and then precipitated with a 2-fold volume of cold ethanol to recover the DNA fragment. It was dissolved again in TE buffer, then cleaved with SalI and subjected to low-melting agarose gel electrophoresis to recover a DNA fragment containing a region coding for the N-terminal of luciferase derived from Luciola cruciata. In this region, a thermostable mutation (Thr217Ile) derived from pGLf37 T-M-2 was contained.

50 ng of the above about 0.5-kbp EcoRV-SalI fragment derived from pT3/T7-LUC and 50 ng of the above about 4-kbp SalI-cleaved fragment derived from pGLf37 T-M-2 were incubated at 15 ℃ for 16 hours in 20 μl DNA ligase buffer in the presence of 300 U of T4 DNA ligase. The reaction mixture was used to transform E. coli JM109 (Toyobo Co., Ltd.) by the Hana-han method [DNA Cloning, 1,

109-135 (1985)], and ampicillin-resistant colonies were selected.

A plasmid was removed from the formed colonies by the alkali-SDS method, This plasmid was subjected to reaction with a dye primer tuck sequencing kit (Applied Biosystems) and analyzed by electrophoresis with an ABI373 DNA sequencer (Applied Biosystems) to determine its nucleotide sequence. The determined nucleotide sequence is shown in SEQ ID NO:8, and the amino acid sequence of a polypeptide translated from said nucleotide sequence is shown in SEQ ID NO:7. The plasmid thus obtained was designated pGGA1.

Plasmid pGGA1 was used to transform E. coli JM109 in the manner described above to give E. coli JM109 (pGGA1). E. coli JM109 (pGGA1) was deposited as FERM BP-5989 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan. E. coli JM109 (pGGA1) was inoculated on an LB-amp agar plate [1 % (W/V) Bacto-trypton, 0.5 % (W/V) yeast extract, 0.5 % (W/V) NaCl, (50 $\mu$ g/ml) ampicillin and 1.4 % (W/V) agar] and cultured at 37 ℃. The colonies appearing 16 hours thereafter were cultured at 37 ℃ for 18 hours under shaking in 10 ml of an LB-amp medium [1 % (W/V) Bacto-trypton, 0.5 % (W/V) yeast extract, 0.5 % (W/V) NaCl and (50 $\mu$ g/ml) ampicillin]. This culture, 10 ml, was inoculated into 2 L of the above LB-amp medium and cultured at 30 ℃ for 6 hours under shaking and then centrifuged at 8000 r.p.m. for 10 minutes to give 30 g wet microbial pellet. The recovered microorganism was suspended in 20 ml buffer consisting of 0.1 M $KH_2PO_4$ (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol and 0.2 mg/ml protamine sulfate, and 2 ml of 10 mg/ml lysozyme solution was further added thereto and the mixture was allowed to stand on ice for 15 minutes. Then, this suspension was frozen in an ethanol/dry ice bath and then allowed to stand at

a temperature of 25 ℃ until it was completely thawed. Further, it was centrifuged at 12,000 r.p.m. for 5 minutes whereby 20 ml crude enzyme was obtained as the supernatant. The crude enzyme solution thus obtained was purified according to the method described in Japanese Patent Laid-Open Publication 141592/1989, and the purified enzyme was designated GGA1 luciferase.

The Km value of this purified GGA1 enzyme for the substrate ATP was determined (Table 2). The result indicated that the affinity of the GGA1 luciferase for ATP was about 1.46 fold higher than the wild-type Photinus pyralis luciferase and about 2.89-fold higher than the wild-type Luciola cruciata luciferase. This improvement in the affinity of the GGA1 luciferase for ATP as compared with that of the wild-type luciferase reveals that the GGA1 luciferases is a very useful enzyme.

Table 2

| | Km (mM) |
|---|---|
| Photinus pyralis luciferase | 0.152 |
| Luciola cruciata luciferase | 0.301 |
| GGA1 luciferase | 0.104 |

This purified enzyme was examined for thermal stability where the remaining activity after treated at 50 ℃ in 0.05 M potassium phosphate buffer (pH 7.8) with 10 % ammonium sulfate saturation was determined. The result indicated that this enzyme maintained 80 % or more of the original activity even after treatment at 50 ℃ for 20 minutes, and it was thus found that the thermal stability of this enzyme has been improved as compared with that of the wild-type Photinus pyralis luciferase and the thermostable Luciola cruciata luciferase.